

Interaction Between Paracrine Tumor Necrosis Factor- α and Paracrine Angiotensin II During Myocardial Ischemia

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OBJECTIVES	The purpose of this study was to explore interactions between paracrine angiotensin II (Ang-II) and tumor necrosis factor- α (TNF- α) during myocardial ischemia.
BACKGROUND	Ischemic myocardium releases significant amounts of TNF- α . This paracrine release correlated with postischemic myocardial injury. Other studies showed myocardial protection obtained by the use of angiotensin-converting enzyme inhibitors (i.e., captopril) and the Ang-II type 1 receptor antagonist losartan after ischemia. The possibility that these agents decrease TNF- α synthesis has not yet been investigated.
METHODS	Using the modified Langendorff model, isolated rat hearts underwent either 90 min of nonischemic perfusion (control group) or 1 h of global cardioplegic ischemia. In both groups, either captopril (360 μ mol/liter) or losartan (182.2 μ mol/liter) was added before ischemia. The hearts were assayed for messenger ribonucleic acid (mRNA) expression and effluent TNF- α levels. In addition, cardiac myocytes were incubated in cell culture with Ang-II.
RESULTS	After ischemia, TNF- α mRNA expression intensified from 0.63 ± 0.06 (control group) to 0.92 ± 0.12 ($p < 0.03$), and effluent TNF- α levels were 711 ± 154 pg/ml. The TNF- α mRNA expression declined to 0.46 ± 0.07 ($p < 0.01$) and 0.65 ± 0.08 ($p < 0.02$) in captopril- and losartan-treated hearts, respectively. Effluent TNF- α was below detectable levels. Concentrations of TNF- α in supernatants of incubated cardiac myocytes treated with 10 and 50 nmol/liter of Ang-II were 206.0 ± 47.0 pg/ml and 810 ± 130 pg/ml, respectively ($p < 0.004$). When pretreated with 700 μ mol/liter of losartan, TNF- α was below detectable levels.
CONCLUSIONS	This study presents an original explanation for previously reported myocardial protection after ischemia, obtained by the use of captopril and losartan. These drugs reduce TNF- α synthesis, providing strong evidence of active interactions between paracrine TNF- α and Ang-II in the evolution of the ischemic cascade. (J Am Coll Cardiol 2001;37:316–22) © 2001 by the American College of Cardiology

There is increasing evidence of the existence of an endogenous and functionally integrated renin-angiotensin system (RAS) in the heart (1,2). Intracardiac angiotensin II (Ang-II) might be involved in the regulation of coronary blood flow, the modulation of sympathetic neurotransmission and cardiac contractility, the stimulation of hyperplasia and hypertrophy and the maintenance of cardiovascular structure and repair (1,2). Angiotensin II interacts with at least two pharmacologically distinct subtypes of cell-surface receptors—Ang-II type 1 and type 2 receptors (3,4). The role of the type 2 receptor is not yet well defined. The type 1 receptor mediates the major cardiovascular effects of Ang-II (5), and its messenger ribonucleic acid (mRNA) expression was found in cardiac myocytes of the human heart (6).

The role of RAS in the pathogenesis of myocardial

ischemia-reperfusion injury has been postulated on the basis of the cardioprotective effects observed with angiotensin-converting enzyme (ACE) inhibitors (7). In several experimental studies, administration of Ang-II caused cardiac myocyte necrosis or induced myocardial infarction (8,9). The ACE inhibitor captopril improved the recovery of isolated rat hearts subjected to ischemia-reperfusion (10). Furthermore, high doses of the Ang-II type 1 receptor-blocking agent losartan, given in cardioplegia before ischemia, significantly improved myocardial recovery of the ischemic-reperfused isolated rat hearts (11).

Tumor necrosis factor- α (TNF- α) is a proinflammatory cytokine that has been implicated in the pathogenesis of cardiovascular diseases (12–16) and myocardial ischemia-reperfusion injury (17). Significant amounts of TNF- α were detected in the effluent of the isolated rat hearts (18,19) having similar degrees of cardioplegic arrest and prolonged ischemia, as shown in captopril- and losartan-treated hearts. Paracrine TNF- α release was shown to play an important role in ischemic injury, as neutralization with specific monoclonal antibodies against this cytokine completely eliminated TNF- α from the effluent

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Manuscript received August 13, 1999; revised manuscript received July 24, 2000, accepted September 13, 2000.

Abbreviations and Acronyms

ACE	= angiotensin-converting enzyme
Ang-II	= angiotensin II
cDNA	= complementary deoxyribonucleic acid
GAPD	= glyceraldehyde-3-phosphate dehydrogenase
LV	= left ventricular
mAb	= monoclonal antibody
mRNA	= messenger ribonucleic acid
PCR	= polymerase chain reaction
RAS	= renin-angiotensin system
TNF-alpha	= tumor necrosis factor-alpha

and concurrently attenuated the postischemic myocardial injury (20).

The purposes of this study were to examine the possible interactions between the aforementioned pathways—Ang-II and TNF-alpha—in the development of myocardial ischemia and to examine how captopril and losartan affect the synthesis and release of TNF-alpha in ischemic, isolated rat hearts. The effect of Ang-II on TNF-alpha production was also studied in cultured rat myocyte cells.

METHODS

Male Wistar rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg body weight) and heparinized. Their hearts were rapidly excised, immersed in cold saline (4°C) with heparin, mounted on the stainless-steel cannula of a modified Langendorff apparatus and perfused with Krebs-Henseleit solution exactly as described in four of our previously reported studies (10,11,18,20).

Left ventricular (LV) hemodynamic variables (peak systolic developed pressure, first derivative of rise of left ventricular pressure (dp/dt_{max}), time-pressure integral and coronary blood flow) were continuously recorded, and measurements were taken at 10-min intervals to confirm functional similarity of myocardial mechanical performance between different groups of isolated rat hearts.

Protocol. Control measurements were recorded after a 15-min period of stabilization, and the hearts were assayed at this point (group A) to effluent TNF-alpha ($n = 7$) and LV TNF-alpha mRNA expression ($n = 4$). The other hearts were then perfused for a 30-min period. Effluent for

TNF-alpha levels were withdrawn at this point ($n = 7$). Warm cardioplegia was then initiated for 2 min (37°C, perfusion pressure 73 mm Hg, 16 mEq/liter KCl in Krebs-Henseleit solution), and a 60-min period of global ischemia at 31°C was applied to the arrested hearts (group B). Other groups were similar to group B but received captopril (360 μ mol/liter, group C), losartan (182.2 μ mol/liter, group D) or monoclonal hamster antimurine TNF-alpha antibodies (anti-TNF-alpha monoclonal antibody [mAb]) in the cardioplegic solution (0.5 μ g/ml, total given dose of 15 μ g, group E). Concentrations of captopril, losartan and anti-TNF-alpha mAb were sufficient, in previous studies, to induce a significant improvement in postischemic LV dysfunction (10,11,20). In groups B to E, at the end of ischemia, the first milliliter of reperfusion effluent was withdrawn to determine TNF-alpha levels ($n = 7$ in each group). Another four or five hearts in each group (B to E) were withdrawn for LV TNF-alpha mRNA assays immediately at the end of ischemia. Group F consisted of isolated hearts undergoing a 90-min period of normal nonischemic perfusion and served as additional control group ($n = 7$) to postischemic TNF-alpha measurements.

Ribonucleic acid isolation and polymerase chain reaction amplification. Immediately after 1 h of global cardioplegic ischemia, LV myocardium was excised and placed in cold Hank's balanced solution. Total RNA was extracted from myocardial samples using the guanidinium thiocyanate method (21). Pellets of RNA were kept at -20°C with 75% ethanol until assay. Dried sediments were dissolved in sterile RNase free water and quantitized spectrophotometrically at wave length 260 nm.

Two micrograms of total RNA were subjected to reverse transcription reaction in 20 μ l using a reverse transcription system (Promega). After completion of the reaction, 5 μ l of this reaction mixture was used for TNF-alpha complementary deoxyribonucleic acid (cDNA) polymerase chain reaction (PCR) amplification, and 5 μ l of 1:10 diluted reaction mixture was used for glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA amplification. Our PCR negative control contained water instead of cDNA, and the cDNA negative control contained water instead of RNA. For TNF-alpha cDNA amplification, the following primers were used: sense—CACGCTCTTCTGTCTACTGA; antisense—GGACTCCGTGATGTCTAAGT, produc-

Table 1. Baseline Measurements

	Group A	Group B	Group C	Group D	Group E	Group F
Peak systolic pressure (mm Hg)	124 \pm 3.2	120 \pm 9.2	117 \pm 4.2	119 \pm 4.4	121 \pm 6.2	119 \pm 5.7
dp/dt_{max} (mm Hg/s)	4,116 \pm 360.8	4,076 \pm 283.1	4,099 \pm 185.3	4,115 \pm 208.2	4,039.0 \pm 169.40	4,045 \pm 173.1
Time-pressure integral (mm Hg \times s)	8.84 \pm 0.69	8.37 \pm 0.38	8.16 \pm 0.44	8.51 \pm 0.53	7.95 \pm 0.47	8.23 \pm 0.59
Coronary blood flow (ml/min)	18.5 \pm 0.93	18.0 \pm 0.85	18.8 \pm 1.14	19.4 \pm 1.32	20.3 \pm 1.54	18.5 \pm 1.26

Data are presented as the mean value \pm SEM. All variables were identical for all groups of hearts according to the Student unpaired *t*-test ($p = NS$).
 dp/dt_{max} = first derivative of rise of left ventricular pressure.

Table 2. Left Ventricular Hemodynamic Variables of Isolated Rat Hearts Measured During the Preischemic Perfusion Period

Time/Variable	Peak Systolic Developed Pressure (%)				dP/dt _{max} (%)			
	Group B	Group C	Group D	Group E	Group B	Group C	Group D	Group E
Baseline	100	100	100	100	100	100	100	100
10-min Perfusion	98.39 ± 1.54	99.87 ± 2.41	97.39 ± 1.56	96.78 ± 2.63	99.13 ± 2.01	99.75 ± 3.65	98.17 ± 1.87	96.25 ± 2.19
20-min Perfusion	96.24 ± 2.45	98.45 ± 1.94	95.32 ± 3.25	94.20 ± 1.26	97.75 ± 2.56	98.87 ± 2.87	98.01 ± 3.58	95.18 ± 2.87
30-min Perfusion	93.13 ± 1.63	95.14 ± 2.26	93.13 ± 2.86	93.47 ± 1.65	95.48 ± 2.18	98.13 ± 2.21	95.26 ± 4.12	94.21 ± 3.48

Data are presented as the mean value ± SEM. Baseline values (at the end of 15 min of stabilization) were considered to be 100%. By two-way analysis of variance: Peak systolic developed pressure—group means: $F = 0.57$, $p = 0.641$; group-time: $F = 1.02$, $p = 0.435$. dP/dt_{max}—group means: $F = 0.20$, $p = 0.898$; group-time: $F = 0.56$, $p = 0.822$. Time-pressure integral—group means: $F = 0.96$, $p = 0.434$; group-time: $F = 0.82$, $p = 0.603$. Coronary blood flow—group means: $F = 0.14$, $p = 0.937$; group-time: $F = 0.62$, $p = 0.776$.

dP/dt_{max} = first derivative of rise of left ventricular pressure.

ing a 546-base pair (bp) fragment (22). An annealing temperature of 57°C was chosen for this reaction. For GAPC cDNA amplification, these primers were used: sense—AATGCATCCTGCACCACCA; antisense—GTAGCCATATTCATTGTCATA, producing a 515-bp fragment (23). The annealing temperature here was 60°C. The optimal cycle number for TNF-alpha was 30. For PCR amplification and reverse transcription reaction, mini-cycler TM (MJ Research Inc., Watertown, Massachusetts) was used. Similar methods were used to determine TNF-alpha mRNA expression in cultured cardiac myocytes.

Quantitative analysis. The PCR products (10 µl) were separated in 1.8% agarose gel, stained with ethidium-bromide, visualized by ultraviolet irradiation and photographed with Polaroid film. The film was taken to evaluate band densities, using the Fujifilm Thermal Imaging System FTJ-500, computer-based Image Capture Software (Pharmacia Biotech, Jerusalem, Israel) and TINA program package (Raytest Isotope Messgerate, GmbH, Staubenhardt, Germany). The intensities of the bands were expressed in arbitrary densitometry units. All TNF-alpha band intensities were normalized by their respective GAPD values. Each PCR reaction was performed at least twice, and four or five hearts were used for each experimental group.

Determination of TNF-alpha. Effluent samples for TNF-alpha measurements were withdrawn at baseline measurements (15' of stabilization), after 30' of perfusion (groups B to F), immediately after ischemia (first milliliter, groups B to E) and at the end of 90 min of normal nonischemic perfusion (group F) and were immediately stored at -70°C until assay. Levels of TNF-alpha were determined on the basis of cytotoxic activity bioassay on mouse L929 cells, according to the methods described by Wallach (24). Each assay included a standard curve of recombinant human TNF-alpha (specific activity 2.5×10^7 IU/mg protein; 1 IU = 40 pg TNF-alpha). The limit of detection was 40 pg/ml.

All effluent cytotoxic activity was neutralized by preincubation with antimurine TNF-alpha mAb: 33.5 ng of antibodies completely neutralized 40 pg of effluent TNF-alpha ($n = 7$), whereas 65.7 ng of antibodies provided $84 \pm 5\%$ of complete neutralization ($n = 6$, $p < 0.002$), thus confirming that the cytotoxic activity was caused by TNF-alpha.

Antibodies. Hamster antimurine TNF-alpha monoclonal antibodies were purchased from Genzyme (Cambridge, Massachusetts). Specific activity: approximately 50 to 100 ng of this antibody will completely neutralize one unit of murine TNF-alpha. Monoclonal hamster antimurine TNF-alpha was previously recognized to completely neutralize rat TNF-alpha (25).

Rat cardiac myocyte cultures. The hearts from neonatal Wistar rats (two to three days old) were removed and finely minced. The pieces were immersed in a dissociation solution (calcium- and magnesium-free Hank's balanced salt solution, Gibco, Basel, Switzerland) containing 1:200 dilution of dissociation enzyme (Institute of Biology, Nes-Ziona, Israel). One million cells were placed in 0.1% gelatin precoated, 35-mm tissue culture plates (Corning). The medium was replaced the following day. Cardiac myocyte cultures were treated with cytosine arabinoside (3 mmol/liter) one day after seeding for two days to abort the multiplication of fibroblasts and other dividing cells, but with no effect on cardiac myocytes, which were essentially postmitotic. Thus, the experiments were done on highly rich cardiac myocyte cell populations (26). Using the periodic acid Schiff procedure (27), the amount of fibroblasts in the cardiac myocytes cultures never exceeded 10%.

Cardiac myocytes were treated with Ang-II on the fourth day after seeding, when the cultures were confluent. Fresh medium was introduced, and Ang-II (10 nmol/liter and 50 nmol/liter) was added for 1 h to study TNF-alpha production in the supernatant. In addition, two other groups of experiments were performed, where losartan (350 and 700 µmol/liter) was given to the cardiac myocytes as a supplement 10 min before Ang-II administration. Supernatant of untreated cardiac myocytes served as a control group.

Ethics. All animals received humane care as described in the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals," prepared by the National Academy of Sciences and published by the National Institutes of Health, Bethesda, Maryland (NIH publication no. 80-23, revised 1985).

Statistics. Results are presented as the mean value ± SEM. The unpaired Student *t* test was used to assess significant differences between groups. To reveal possible differences in

Table 2. Continued

Time-Pressure Integral (%)				Coronary Blood Flow (%)			
Group B	Group C	Group D	Group E	Group B	Group C	Group D	Group E
100	100	100	100	100	100	100	100
99.25 ± 2.53	98.13 ± 1.68	96.57 ± 2.15	95.34 ± 2.71	100.54 ± 5.13	99.76 ± 3.12	98.31 ± 2.13	97.95 ± 4.06
94.67 ± 4.08	97.30 ± 1.94	91.03 ± 3.15	94.78 ± 1.83	96.07 ± 4.76	95.48 ± 2.37	96.45 ± 3.15	95.12 ± 2.16
90.37 ± 2.65	92.36 ± 2.03	89.36 ± 3.40	93.35 ± 4.50	94.70 ± 4.28	95.17 ± 4.02	94.41 ± 2.54	94.56 ± 3.48

each of the LV hemodynamic variables in the experimental groups before cardioplegic ischemia (baseline values: 10, 20 and 30 min perfusion), two-way analysis of variance with repeated measures was performed, comparing group mean values and assessing the interaction between group and time. Significance was established at $p < 0.05$. All statistical analyses were performed using the SPSS computer program (SPSS Inc., Chicago, Illinois) by the Statistics Department of our medical center.

RESULTS

The baseline values (groups A to F) for different LV hemodynamic variables are given in Table 1. No significant differences were found between any experimental groups. Two-way analysis of variance (comparing group mean values and group-time interaction) was done to reveal possible differences in LV hemodynamic variables in the experimental groups (groups B to E) before cardioplegic ischemia (baseline values: 10, 20 and 30 min perfusion). The data are given in Table 2. None of the p values attained statistical significance.

Effect of captopril and losartan on myocardial TNF-alpha mRNA production in ischemic hearts. The expression of mRNA for TNF-alpha was analyzed by using reverse transcription PCR primers that were shown to amplify the reverse-transcribed mRNA in rat hearts. Basal TNF-alpha mRNA expression was detected in LV specimens after a period of stabilization and did not change after a 90-min period of nonischemic perfusion (Fig. 1A). The intensities of the bands at baseline and at the end of perfusion were 0.52 ± 0.05 and 0.63 ± 0.06 , respectively ($p = \text{NS}$) (Fig. 1B).

After 1 h of global cardioplegic ischemia, TNF-alpha mRNA expression increased to 0.92 ± 0.12 , which was significantly higher than those levels detected in nonischemic, normally perfused hearts ($p < 0.03$) (Fig. 1).

When either captopril (360 $\mu\text{mol/liter}$) or losartan (182.2 $\mu\text{mol/liter}$) was added to the cardioplegic solution, normalization of TNF-alpha mRNA expression was observed after 1 h of global cardioplegic ischemia (Fig. 1A). The intensities of the bands were significantly lower than those in the ischemic group, measured after 1 h of global cardioplegic ischemia (group C: 0.46 ± 0.07 [$p < 0.01$], group D: 0.65 ± 0.08 [$p < 0.02$] and group B: 0.92 ± 0.12 , respectively), and there were no differences in the intensities

of these bands as compared with the bands of the nonischemic, normally perfused hearts (Fig. 1B).

Effect of captopril and losartan on postischemic myocardial TNF-alpha release. Tumor necrosis factor-alpha was not detected in the myocardial effluent after 15 min of stabilization, before ischemia (after 30 min of perfusion) and after 90 min of normal, nonischemic perfusion. Significant amounts of TNF-alpha were detected in the effluent after 1 h of global cardioplegic ischemia during the first minute of reperfusion (711 ± 154 pg/ml). When captopril (360 $\mu\text{mol/liter}$) or losartan (182.2 $\mu\text{mol/liter}$) was added to the cardioplegic solution in similar ischemic conditions, effluent TNF-alpha was below detectable levels (<40 pg/ml).

Effect of TNF-alpha depletion on myocardial TNF-alpha mRNA production. To neutralize TNF-alpha in the ischemic heart, we added anti-TNF-alpha mAb to the cardioplegic solution. After 1 h of global ischemia in hearts treated with anti-TNF-alpha mAb, TNF-alpha mRNA

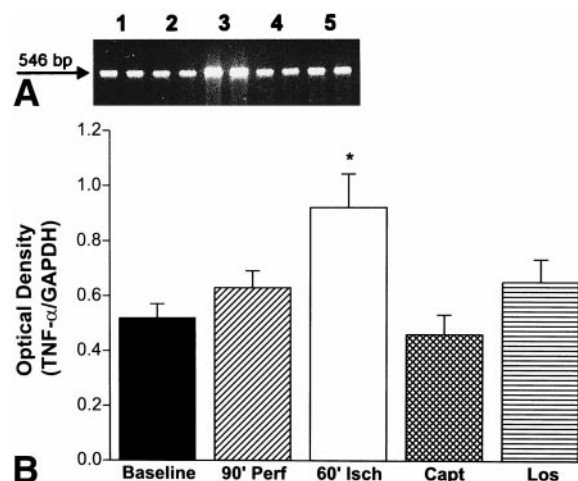


Figure 1. Effects of captopril and losartan on myocardial TNF-alpha mRNA expression in ischemic hearts. **A**, Representative PCR analysis of RNA samples (in duplicate). **B**, Relative optical density of TNF-alpha PCR signal. Data were normalized to relative GAPD PCR signal. Samples withdrawn at the beginning of perfusion ($n = 5$) (baseline) and after 90 min of normal, nonischemic perfusion ($n = 4$) (90' Perf) served as the control groups. In hearts undergoing global cardioplegic ischemia ($n = 5$), TNF-alpha mRNA expressions increased ($*p < 0.03$) (60' ischemia). A significant decrease in TNF-alpha mRNA expression is observed in hearts receiving either captopril (360 $\mu\text{mol/liter}$, $n = 5$) (Capt) or losartan (182.2 $\mu\text{mol/liter}$, $n = 5$) (Los) in cardioplegic solution, before ischemia. Band intensities were significantly lower than those of the ischemic group ($p < 0.01$ and 0.02 , respectively).

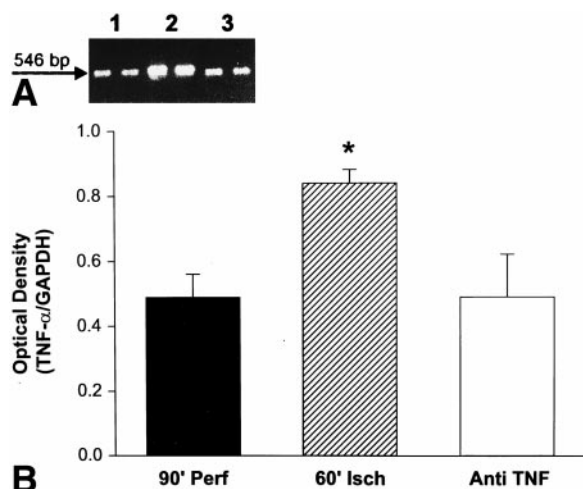


Figure 2. Effect of TNF-alpha depletion on myocardial TNF-alpha mRNA expression in the ischemic rat heart. **A**, Representative PCR analysis of RNA samples (in duplicate). **B**, Relative optical densities of TNF-alpha PCR signal. Data were normalized to relative GAPD PCR signal. 90' Perf = 90 min of normal nonischemic perfusion, serving as control group (n = 4); 60' Isch = hearts undergoing 1 h of global cardioplegic ischemia (n = 5); Anti TNF = hearts receiving monoclonal anti-TNF-alpha antibodies in the cardioplegia (n = 5) just before ischemia, showing significantly lower TNF-alpha mRNA expression than detected in untreated ischemic hearts (*p < 0.03).

expressions were at basal levels (Fig. 2A), significantly lower than those levels detected in untreated ischemic hearts (0.5 ± 0.13 , $p < 0.03$) (Fig. 2B). Protein production of TNF-alpha in the ischemic hearts treated with anti-TNF-alpha mAb was below detectable levels.

Effect of Ang-II on TNF production in rat cardiac myocyte culture. To determine whether Ang-II can promote TNF-alpha protein production, we incubated cardiac

myocyte cultures with Ang-II in concentrations of 10 and 50 nmol/liter for 1 h. Using bioassay of L929 cells, 206.0 ± 47.0 pg/ml and 810 ± 130 pg/ml of TNF-alpha were detected in the supernatants of the 10 and 50 nmol/liter Ang II, respectively ($p < 0.004$). In the supernatants of untreated cardiac myocyte culture, TNF-alpha was below detectable levels (Fig. 3), and the intensity of TNF-alpha mRNA bands was 1.0 ± 0.05 . After 1 h of incubation with 50 nmol/liter of Ang-II, the intensity increased to 1.25 ± 0.03 ($p < 0.01$).

When pretreated with 350 μ mol/liter of losartan, the TNF-alpha concentration in the supernatants of cardiac myocytes incubated with 10 and 50 nmol/liter of Ang-II was below detectable levels and 109.3 ± 25.4 pg/ml ($p < 0.006$), respectively. After pretreatment with 700 μ mol/liter of losartan, TNF-alpha was below detectable levels in both supernatants (Fig. 3).

DISCUSSION

Two mechanisms—a common pathway to myocardial ischemic injury. Angiotensin II plays a pivotal role in myocardial ischemic injury (8,9,28–30). Our results suggest that the effect of Ang-II on myocardial ischemic injury is mediated by a paracrine pathway that increases synthesis and release of TNF-alpha from myocytes. This paracrine release of TNF-alpha is correlated with the postischemic deterioration of LV function (18).

Elevated TNF-alpha, a proinflammatory polypeptide hormone, has a potent negative inotropic effect (1,31,32). Recently, we (18) and others (22) found that TNF-alpha is released from ischemic hearts. In the present study, we also

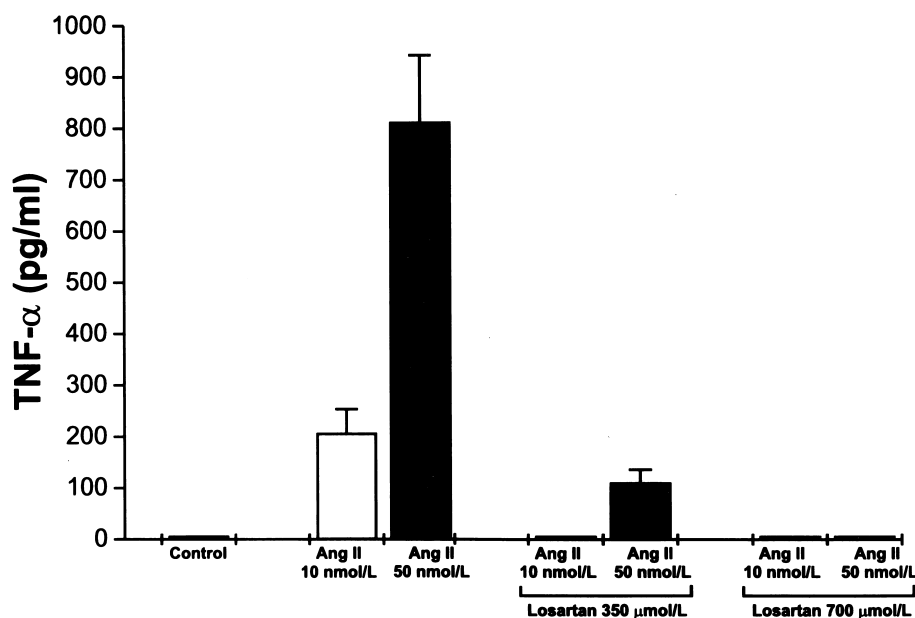


Figure 3. Effect of losartan on Ang-II-induced TNF-alpha production in rat cardiac myocyte culture. No TNF was measured in the supernatant of untreated cultures, serving as the control group (n = 5). Incubation with 10 and 50 nmol/liter of Ang-II caused significant, dose-dependent TNF-alpha release from myocytes. Preincubation with 350 or 700 μ mol/liter of losartan (n = 5 in each group) decreased or abolished the observed Ang-II-induced TNF-alpha release.

found an increase of TNF-alpha mRNA expression, as found by others (22,33). In our pilot study, we documented that 15 min of global ischemia was not sufficient to induce TNF-alpha synthesis and release from ischemic hearts (unpublished data).

Angiotensin II and TNF-alpha in ischemic myocardial damage. High doses of captopril (10), an ACE inhibitor, and losartan, a specific Ang-II type 1 receptor blocker (11,34), given before ischemia, improved recovery of ischemia-reperfusion injury of isolated rat hearts. In this study, both captopril and losartan inhibited the synthesis of TNF-alpha mRNA, as well as heart perfusate effluent TNF levels. The decrease in TNF-alpha synthesis from the ischemic hearts by captopril and losartan may explain the reduction in myocardial damage (10,11).

The exact mechanism of this effect is not well established. In the current study, Ang-II-induced TNF-alpha release from cardiac myocytes through Ang-II type 1 receptors was documented, as losartan abolished this effect.

Both TNF-alpha and Ang-II are probably linking milestones in the evolution of ischemic injury. Tumor necrosis factor-alpha is involved in the release of free radicals from the myocardium (35)—a self-amplifying process—as production of free radicals has been shown to further increase TNF-alpha (36). Both TNF-alpha and Ang-II act through the nitric oxide system and cause myocardial dysfunction after ischemia (37).

Anti-TNF-alpha mAb was found to prevent myocardial dysfunction during experimental burn shock (15), viral myocarditis (14) and allograft rejection (16). In this study, anti-TNF-alpha mAb prevented postischemic paracrine TNF-alpha release from the myocardium and abolished the increase of TNF-alpha mRNA expression in the ischemic hearts.

A new understanding of previous TNF-alpha immunohistochemical studies. Immunohistochemical staining of ischemic hearts localized TNF-alpha to cardiac myocytes and, to a lesser extent, to endothelial cells of vessels (20). Immunostaining of TNF-alpha was not apparent in nonischemic, normally perfused hearts, nor in hearts pretreated with anti-TNF-alpha mAb. The antibodies probably did not cross cell membranes. We therefore hypothesized (20) that anti-TNF-alpha mAb binds TNF-alpha and prevents their attachment to cell membrane receptors. This might be responsible for the self-amplifying process—that is, the further increase in TNF-alpha synthesis during prolonged ischemia. A reasonable explanation for the observed cardioprotective effect of anti-TNF-alpha might be elimination of TNF-alpha from the ischemic cascade, as the entire “snowball” effect is significantly decreased, and therefore ischemic damage is also decreased. When anti-TNF-alpha antibodies were given, no increased TNF-alpha mRNA expression was found. However, factors other than TNF-alpha and Ang-II are probably important to the evolution of ischemic damage (38,39).

Conclusions. In the present study, we found an active interaction between two important pathogenetic pathways—paracrine TNF-alpha and paracrine Ang-II—in the evolution of myocardial ischemic cascade. Captopril, losartan and anti-TNF-alpha mAb were beneficial in ischemia-reperfusion damage of the isolated perfused hearts.

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